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Risk for Sporadic Breast Cancer in Ataxia Telangiectasia Heterozygotes
IDEA Grant # DAMD 17-98-1-8161
Ute M. Moll, M.D.
First Year Progress Report

Scope: The scope of this IDEA grant is to assess whether heterozygosity for the ATM gene, due to a loss of function mutation in one of the 2 alleles and found in about 1% of the general population, confers a significant increase in breast cancer risk for women with sporadic breast cancer (without a family history of breast cancer). This is called the AT - carrier risk hypothesis for sporadic breast cancer.

Introduction: The characterization of BRCA1 and BRCA2 gene mutations as high risk factors in familial breast cancer served as paradigm for the assessment of other more common but less penetrant genes as potential genetic risk factors for sporadic breast cancer. The ATM gene is such a candidate, since AT homozygous patients have (among other symptoms) a cancer phenotype and their cells exhibit excessive radiosensitivity. Moreover, AT heterozygote carriers, which occur at a significant prevalence in the general population, show an intermediate in vitro radiosensitivity, although clinically they are free of AT symptoms. Three epidemiological studies, before the ATM gene was cloned had estimated a relative risk of breast cancer in AT heterozygotes of 3.9 (1-4). However, once the ATM gene was cloned, direct mutational analysis on cumulatively over 500 patients failed to support the hypothesis that a mutant ATM allele plays a role in carcinogenesis and that ATM is a suppressor gene (5-7). Also, in the few cancers which harbored a mutant allele (somatically or constitutionally), no selection pressure exists against the retained wild type allele. Moreover, ATM mutations also failed to correlate with complications in those breast cancer patients with complications after radiotherapy (8, 9). Clearly, these studies show that diagnostic or occupational exposure to ionizing radiation is not enough to increase the relative risk significantly. LOH analysis in the 11q23 region does find a roughly 40% frequency of LOH in the region that includes but does not focus on the ATM locus, thereby leaving it unclear whether the true deletional target is ATM or another unknown suppressor gene(s) thought to reside at this locus.

The overall picture that emerges from cytogenetic and mutational studies on over 1, 200 breast cancers over the past 1 1/2 years is comprehensive and can be summarized as follows. ATM heterozygosity is not a significant genetic determinant in unselected sporadic breast cancer (10-18). All studies fall into one of 2 categories. One category finds a slightly increased risk (that is only minimally higher than the classical reproductive risk factors), while the other category finds no important role of ATM heterozygous mutations in sporadic or even familial breast cancer. Taken together, the studies say that among women with heterozygous ATM mutations there is a) at best a very slightly increased risk for those who come from families with AT syndromes or breast and gastric cancers and b) there is most probably not a measurably increased risk for unselected women.

For this grant, the goals for the first 12 months were:

Aim I Genetic analysis of ATM in clinical samples.

IA) LOH mapping at 11q23.1 in sporadic breast carcinomas using intragenic and ATM flanking microsatellite markers (months 1-18).

Time Table

l LOH mapping at 11q23.1 in breast carcinoma/normal tissue matched pairs using intragenic and ATM flanking microsatellite markers (months 1-18)

l LOH mapping at 11q23.1 in DNA from normal controls using intragenic and ATM flanking microsatellite markers (months 1-18)

Progress in LOH mapping:
Using 6 polymorphic microsatellite markers in and around the ATM locus, we completed LOH analysis on 16 matched breast cancer/normal pairs with the following **results**:

D11S2179 (intragenic ATM):	4 of 16 (25%)
NS22 (intragenic ATM):	3 of 16 (19%)
D11S1787 (centromeric):	4 of 16 (25%)
D11S1778 (telomeric):	6 of 16 (38%)
D11S1294 (telomeric):	6 of 16 (38%)
D11S1818 (telomeric):	4 of 16 (25%)

Interpretation: Our results on frequencies of the ATM and flanking loci in breast cancer is similar to the ones reported in the literature.

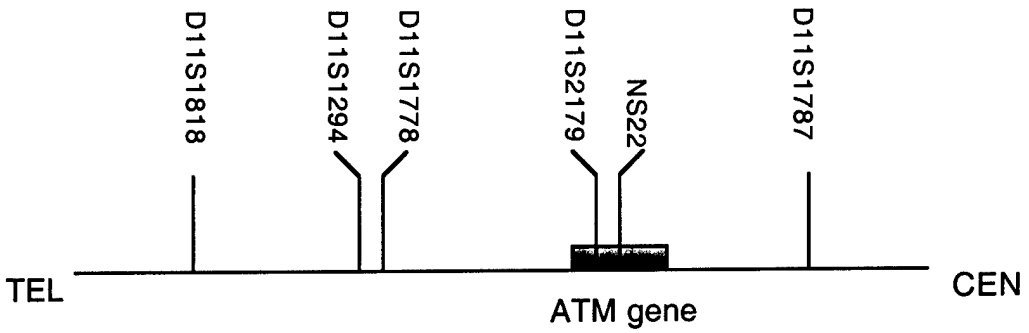

Fig. 1: Genetic markers along 3 Mb genomic DNA spanning the ATM locus on chromosome 11q22-23.

In summary, our LOH results only confirmed the frequency data was already in the literature. Furthermore, the new mutational studies that appeared in the meantime did not show a significant mutational rate of the ATM gene. The latter is a strong but not absolute argument against a true suppressor role of the ATM gene in breast cancer. Rather than simply to continue LOH analysis on the originally planned 145 total cases, we decided to address the question from a different angle. We asked whether the expression status of ATM differed in breast cancers and breast cancer cell lines compared to normal breast tissue. If ATM has a suppressor role in breast cancer, a loss of wild type ATM expression rather than mutational inactivation could be expected.

With this rationale, we undertook a comprehensive ATM expression analysis using quantitative RT-PCR on 89 randomly selected breast cancer samples (from 3 different institutions), 7 breast cancer lines and 29 normal breast samples. Of these, 11 were matched normal/cancer pairs. Our working hypothesis was to find a decreased expression in cancer compared to normal breast tissue.

After obtaining this result, we extended Aim I into analysis of ATM mRNA abundance using an RT-PCR approach from tRNA extracted from tumor and normal breast tissues. We also performed a partial mutational analysis on two regions of the ATM gene (a middle region an dthe PI3 kinase region) on 8 cases of breast cancer with the highest ATM expression.

Results of ATM expression in breast cancer and normal breast tissues. Using a competitive semiquantitative RT-PCR approach, we determined relative ATM expression levels on 89 breast cancers and compared them to 29 normal breast samples (Table 1). Eleven of these constituted matched tumor/cancer pairs. ATM and β2M transcripts were detectable in all breast tissues and the 7 breast cancer cell lines that we analyzed. While the expression of β2M was similar in all samples, ATM expression levels varied widely. Moreover, breast cancer tissues did not show a deficiency in ATM expression. Table I contains the complete set of normalized expression data. Figure 1A shows it graphed as a box plot and Figure 1B shows the same data plotted as a histogram. In fact, cancers expressed mildly higher (1.5-fold) levels of ATM transcripts than normal breast tissues. However, due to the large variance in breast cancers and the relatively small difference between the geometric means of cancer versus normal tissue, the power to detect significant differences between the two groups was very low. The geometric mean of breast cancer was 0.484 +/- 2.5 standard deviation (Std.) compared

to 0.329 +/- 0.30 Std. in normal breast tissue (Fig. 1A). In breast cancer, relative ATM expression ranged from 0.03 to 16.8 with a median of 0.57, and in normal breast it ranged from 0.093 to 1.31 with a median of 0.318. Examples of individual raw data are shown in Fig. 2. Repeat determinations from individual patients yielded reproducible results. Table II shows a subset of breast cancers and normal controls with their relative ATM expression levels, averaged from 2 independent measurements of the same sample. A mild tumor-associated increase in relative ATM transcript levels was also seen when the subgroup of matched pairs was analyzed separately. Seven of the 11 normal / cancer pairs showed a 1.2 to 2.3-fold increase in cancers compared to their adjacent normal tissue match, 3 cases were equal and only one case showed decreased (< 50%) ATM expression in the tumor (Fig. 3). In line with the findings in primary cancers, breast cancer cell lines had even higher ATM expression with a geometric mean of 2.6 +/- 1.96 Std. and a range from 0.47 to 5.55.

Discussion

In this study, we carried out a comparative analysis of ATM expression in 89 unselected sporadic breast cancers and 29 normal breast tissue, of which 11 cases consisted of matched normal/cancer pairs. The study also included 7 breast cancer cell lines. In contrast to our working hypothesis, we find that cancer tissues express relatively higher levels of ATM compared to normal breast tissue (3.2- and 4.1- fold higher mean expression in double and single calculations, respectively). This tumor-specific increase is highest (8- and 10 fold, respectively for double and single calculations, compared to normal breast tissue) in breast cancer cell lines (T47D, MDA435, MDA231, MDA468, MDA 361, SkBr-3 and MCF7). Moreover, of the 11 matched cases, 7 cases showed tumor-specific increase in ATM expression ranging from 1.2 to 2.3-fold compared to adjacent normal breast tissue. Taken together, our results show that breast cancer expresses higher ATM levels on average, regardless whether matched or unmatched comparisons are made and what type of algorithms are used. Furthermore, this effect is independent of previous genotoxic exposure since none of the matched pair patients had received neoadjuvant treatment before surgery. However, since the highest ATM increases were seen in some cases that had received chemotherapy 4 weeks prior to surgery, an triggered DNA damage response might have contributed to ATM induction in some patients. It remains to be proven that the increased ATM levels mean increased enzymatic activity in tumor tissue. However, this is likely to be the case since known ATM mutations, for which we did not test here, that are found in AT patients are mostly truncation mutations spread throughout the entire gene rather than subtle missense mutations. The amplicon used in this study lies in the middle of the ATM open reading frame (nucleotide position 4646-4905 of 9385 nucleotides total). Therefore, it appears unlikely that the majority of our overexpressing samples harbored an unrecognized truncation mutation downstream.

ATM is a member of the phosphatidylinositol-3 kinase family and a so-called 'stress kinase' that is involved in meiotic recombination, telomere length monitoring and γ-IR induced DNA damage response. These cellular programs are frequently altered in tumor cells. P53 and the non-receptor tyrosine kinase c-abl are important downstream signaling targets for ATM. Moreover, ATM plays a role in p53-independent S and G2/M checkpoints. Therefore, a reasonable interpretation of our results is that the inherent genetic instability in tumor cells combined with the multiple checkpoint failures in the tumor cell cycle is sensed as cellular stress that elicits an upregulation of the ATM gene as a compensatory mechanism. Concerning ATM's potential role in causing sporadic breast cancer, our result does not support a suppressor role in this disease. Furthermore, our results suggest that many breast tumors with LOH at the ATM locus (about 30% of breast cancers) might in fact overexpress the gene, rendering a mechanism of haploinsufficiency also unlikely.

Key Research Accomplishments:

-Paper published (Kovalev S et al (2000) International Journal of Oncol 16: 825-831.

-Poster presentation at the Era of Hope Meeting, June 8-11, 2000 in Atlanta

-Our study did not find evidence in support of the hypothesis that ATM is a tumor suppressor gene causally involved in sporadic breast cnacer. Our study agrees with several new studies in the literature which appeared since the proprosal was originally submitted.

Reportable Outcome:

1) This work was published: Kovalev S, Mateen A, Zaika AI, O'Hea BJ and UM Moll (2000) Lack of defective expression of the ATM gene in sporadic breast cancer tissues and cell lines. *Int J Oncol* **16**: 825-831.

2) A repository of total RNA extracted from the 89 cases of breast cancer and 29 cases of normal breast tissue has been made an dwill be availabe for future molecular studies.

3) Based on the experience and training received from the work supported by this award, Dr. Sergey Kovalev obtained a faculty position in his native country at the University of Yekaterinburg, Russia. There, he is working in the field of molecular diagnosis of malignancies, including breast cancer.

Conclusions:

Although the ATM locus falls within a region of frequent LOH in breast and other human cancers, we did not find a reduction in ATM mRNA expression levels in our cohort of 89 sporadic breast cancers. Based on the available mutations data (mostly truncations leading to unstable protein), such a reduction would be expected at least in some cases if the ATM gene would play a causal role in breast cancer. Our results do not support such a suppressor role for ATM in the development of sporadic breast cancer.

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Lack of defective expression of the ATM gene in sporadic breast cancer tissues and cell lines

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Abstract. Homozygous mutations of the gene mutated in ataxia telangiectasia (ATM) causes the AT syndrome, a pleiotropic phenotype that includes an increased risk of cancer. Most of the known mutations at the ATM gene lead to truncations which are usually associated with instability of mRNA and protein. A decrease or loss of ATM protein expression is associated with specific lymphoid malignancies in AT and non-AT patients. ATM is located within a region in chromosome 11q22-23 that is frequently undergoing loss of heterozygosity in sporadic breast cancer. Epidemiological studies estimated a 4-fold increase in breast cancer risk in heterozygous women. However, direct mutational analysis failed to clearly support a role for mutant ATM alleles in breast carcinogenesis. If ATM does have a suppressor role in this tissue, one would expect deficient ATM expression. We therefore tested the hypothesis that the expression of the ATM gene is reduced in sporadic breast cancer. We determined ATM transcript levels using competitive RT-PCR on 89 randomly selected sporadic breast cancer samples and 29 normal breast tissues. Of these, 11 were matched normal/cancer pairs. We also evaluated 7 breast cancer cell lines. Deficiency in ATM expression was not observed. Of the 11 matched pairs, 7 tumors expressed mildly higher levels, 3 tumors expressed the same amount and only 1 tumor expressed <50% of the normal match. In addition, 3 cancers with tumor-associated LOH of the ATM gene expressed higher mRNA levels in the tumors than in their normal tissue matches, suggesting that no correlation exists between tumors with LOH and decreased ATM expression. In summary, our results do not support a suppressor role for ATM in the development of sporadic breast cancer.

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Abbreviations: LOH, loss of heterozygosity; T-PLL, T-cell prolymphocytic leukemia; B-CLL, B-cell chronic lymphocytic leukemia; β2M, β2-microglobulin

Key words: ATM gene, expression, loss of heterozygosity, mutation, breast cancer

Introduction

The characterization of BRCA1 and BRCA2 gene mutations as high risk factors in familial breast cancer served as paradigm for the search for other more common but less penetrant genes as potential risk factors for sporadic breast cancer. The ATM gene is such a candidate, since AT homozygous patients have, among many other symptoms, an increased frequency of lymphoid malignancies and their cells exhibit excessive radiosensitivity. AT heterozygote carriers occur at a prevalence of 0.5-1% in the general population but are clinically free of AT symptoms. However, their cells show an intermediate radiosensitivity *in vitro* as well as a defective control of the mitotic spindle checkpoint after X-rays, although a defect in apoptosis is controversial (3,4). Before the ATM gene was cloned in 1995, 4 epidemiological studies had estimated a relative risk of breast cancer in AT heterozygotes of 3.9 (5-8). LOH analysis of sporadic breast cancer on chromosome 11q23 shows up to 40% frequency of loss of heterozygosity spanning an approximately 6 Mb region that includes the ATM locus (9,10). This leaves it unclear whether the true deletional target is ATM and/or another unknown suppressor gene(s) thought to reside in this region (9,11). Consistent with this data, LOH of ATM using the intragenic marker D11S2179 and the distal marker D11S1818 are associated with poorer survival (11). However, since the ATM gene has been cloned, direct mutational analysis of mainly constitutive DNA but also tumor DNA on cumulatively over 500 breast cancer patients from non-AT families, failed to clearly support the hypothesis that a mutant ATM allele plays a role in breast cancer risk/development and that ATM is a *bona fide* suppressor gene in this tissue (12-15). This includes a failure to detect increased germline mutations in the ATM gene of women with early onset (<40 years) sporadic breast cancer (12) and of women from breast and gastric cancer families, which appear to be the most frequent malignancies seen in AT carriers (13,14). Also, in the few breast cancers analyzed to date which harbored a mutant ATM allele somatically or constitutionally, no selection pressure appears to exist against the retained wild-type allele in the tumor (14,15). ATM mutations and constitutional ATM heterozygosity also failed to correlate with complications after radiotherapy in breast cancer patients with an adverse reaction (16-18). Clearly, these studies show that diagnostic or occupational exposure to ionizing radiation is not enough to increase the relative risk of breast cancer significantly (19).

The overall picture that emerges to date from LOH and mutational studies on over 1,200 breast cancers indicates that the connection between AT heterozygosity and breast cancer remains unclear. The difficulty in proving a connection, however, suggests that AT heterozygosity is not a significant genetic determinant in unselected sporadic breast cancer (12-15,19,20). Furthermore, the studies suggest that among women with heterozygous ATM mutations there is: i) at best a slightly increased risk for those from families with AT syndromes (20,21) or from select families with breast cancer, leukemias and lymphomas (13,14,21).

Although direct mutational studies in breast cancer did not show a significant mutational rate of the ATM gene, the data, albeit strong, is not an absolute argument against a genuine suppressor role of the ATM gene in sporadic breast cancer. Haploinsufficiency, particularly in light of a significant LOH status at and around the ATM locus, or epigenetic modes of downregulating the expression could in theory be alternate mechanisms. Importantly, a decrease or loss of ATM protein expression due to mutational inactivation is associated with specific lymphoid malignancies in AT and non-AT patients. ATM expression is decreased in the rare T-cell prolymphocytic leukemia (T-PLL) that occurs in young AT patients (22) or in older patients due to somatic loss of both alleles (23) and in an aggressive subgroup of B-cell chronic lymphocytic leukemia (B-CLL) with LOH at the ATM locus (24,25). We therefore tested the hypothesis that the expression of the ATM gene is also reduced in breast cancers and breast cancer cell lines compared to normal breast tissue. If ATM does indeed have a suppressor role in breast cancer, lost or decreased wild-type ATM expression might reflect or substitute for mutational inactivation. So far, one expression analysis on breast cancer has been reported (39 cases), and this study found a reduction in the mean ATM transcript level in carcinomas vs normal breast tissues (26). However, the basis for the reduced expression was unclear since LOH analysis did not include the ATM gene itself and direct analysis of the PI3 kinase region of ATM failed to detect mutations (26). With this background, we undertook a comprehensive ATM expression analysis using competitive RT-PCR on 89 randomly selected sporadic breast cancer samples and 29 normal breast tissues. Of these, 11 were matched normal/cancer pairs. We also included 7 breast cancer cell lines. In this cohort, primary breast cancers and breast cancer lines did not express reduced levels of ATM transcripts compared to normal breast tissue.

Material and methods

Tissues and cell lines. Malignant tissues were obtained from 39 women at University Hospital at SUNY Stony Brook undergoing surgery for breast cancer and from 57 additional breast cancer patients through the Cooperative Human Tissue Network, Western Division (Case Western Reserve University, Cleveland OH). All cancers had pathologically confirmed diagnosis. Eighty-three cancers were invasive ductal carcinomas, 9 were ductal carcinoma *in situ* and 4 were invasive lobular carcinomas. Our series also comprised 36 normal breast tissues, 18 of which were matched pairs of cancer and adjacent normal tissue from the same patient while 18 were from unrelated reduction mammoplasties. Of the matched pairs, 11 were used for expression analysis and 16 were used for LOH analysis. Freshly harvested tissues were immediately snap frozen in liquid nitrogen and stored at -80°C until needed. Human breast cancer cell lines MDA 468, MDA 361, MDA 231, MDA 435, MCF-7, T47D and SK-BR-3 were grown in 10% FCS containing DMEM at 5% CO_2.

RNA and DNA extraction. Snap frozen tissue was homogenized under liquid nitrogen in 2 ml of RNA STAT-60 (Tel-Test, Inc. Friendswood TX). After adding chloroform, total RNA was precipitated in isopropanol, washed twice in 75% ethanol and dried. Concentrations of reconstituted RNA were measured in triplicate by UV spectrophotometry and adjusted to 1 µg/ml. To obtain corresponding DNA, DNA reverse extraction from the same samples was performed using DNA STAT-60 (Tel-Test, Inc.).

Competitive RT-PCR. A competitive RT-PCR method was used to determine the amount of ATM transcripts in individual samples as described (26,27). Briefly, a predetermined constant amount of a mutagenized (deleted) homologous competitor RNA was added to 250 ng of individual tumor RNA prior to the RT/PCR reaction (single tube format, Titan Kit, Boehringer/Roche). To make competitor RNA, template cDNA product was generated by a first PCR reaction using forward primer (ATMf) 5'-TGTCATTACGTAGCTTCTCC and reverse primer (ATMr) 5'-GCTGAGTAATACGCAAA TCC (nucleotide positions 4646-4665 and 4924-4905, GenBank #U33841). The reaction was performed using a standard PCR protocol. Amplicon I was subsequently mutagenized by a second PCR reaction that introduced a 5' deletion using the forward competitor primer 5'-TGTCATTACGTA GCTTCTCCacttactgtaaggatgctctag (position 4646-4665/ 4708-4729) and the ATMr primer. For generating RNA, amplicon II was cloned into the pPCR-Script Amp SK(+) cloning vector (Stratagene) followed by *in vitro* transcription (Stratagene RNA transcription kit). Competitor concentration was determined by spectrophotometry. To determine the proper amount of competitor addition, serial dilutions (10 fg to 100 pg) were added to 250 ng of pooled sample RNA and subjected to RT-PCR using primers ATMf and ATMr. Optimal calibration was defined at equal signal intensity between sample and competitor and was chosen for all subsequent individual tumor measurements. To standardize for RT efficiency, expression levels of the housekeeping gene ß2 microglobulin (ß2M) was determined analogously in a separate reaction including generation of a competitor and its calibration (ß2Mf primer 5'-TGTCTTTCAGCAAGGACTGG, ß2Mr primer 5'-GATGCTGCTTACATGTCTCG and ß2M competitor primer 5'-TGTCTTTCAGCAAGGACTGGaaa gatgagtatgcctgccgt). Amplicons were separated on a 6% denaturing acrylamide gel and quantitated by PhosphoImage analysis (model 445 SI, Molecular Dynamics). ATM expression levels were calculated using the competitive algorithm (ATM patient/ATM $_{competitor}$/ß2M $_{patient}$/ß2M $_{competitor}$) and in some cases also the simple algorithm (ATM $_{patient}$/ß2M patient). For statistical analysis, groups were analyzed using the Stastistix program.

Table I. Relative ATM expression in breast cancer tissues.

Breast cancer case #	Relative ATM expression	Breast cancer case #	Relative ATM expression
BC1	0.165	BC47	0.06
BC2	0.25	BC48	0.11
BC3	0.24	BC49	0.25
BC4	1.63	BC50	0.48
BC5	1.08	BC51	0.65
BC6	2.3	BC52	0.37
BC7	0.77	BC53	0.26
BC8	0.61	BC54	0.35
BC9	1.29	BC55	0.05
BC10	0.63	BC56	0.07
BC11	0.89	BC57	0.09
BC12	2.11	BC58	0.08
BC13	1.38	BC59	0.2
BC14	0.44	BC60	0.19
BC15	0.75	BC61	0.1
BC16	0.8	BC62	0.08
BC17	0.68	BC63	0.03
BC18	5.3	BC64	0.04
BC19	0.53	BC65	1.06
BC20	0.36	BC66	4.35
BC21	0.71	BC67	1.12
BC22	1.44	BC68	0.5
BC23	0.96	BC69	0.41
BC24	0.64	BC70	0.08
BC25	1.39	BC71	0.26
BC26	1.76	BC72	0.594
BC27	1.46	BC73	0.186
BC28	0.49	BC74	0.096
BC29	1.15	BC75	0.393
BC30	6.37	BC76	0.195
BC31	0.76	BC77	7.67
BC32	0.73	BC78	1.99
BC33	0.478	BC79	2.78
BC34	0.57	BC80	7.34
BC35	0.87	BC81	2.97
BC36	1.1	BC82	5.84
BC37	2.36	BC83	6.56
BC38	0.052	BC84	16.79
BC39	0.07	BC85	0.479
BC40	0.06	BC86	0.065
BC41	0.483	BC87	0.61
BC42	0.12	BC88	0.05
BC43	0.09	BC89	0.76
BC44	0.46		
BC45	0.07	geoMean	0.484
BC46	0.09	SD	2.5

Table I. Continued.[a]

Normal breast tissue	Relative ATM expression	Normal breast tissue	Relative ATM expression
Normal 1	0.16	Normal 17	0.628
Normal 2	0.215	Normal 18	0.176
Normal 3	0.275	N1 (of BC1)	1.31
Normal 4	0.51	N61 (of BC61)	0.093
Normal 5	0.42	N62 (of BC62)	0.333
Normal 6	0.36	N63 (of BC63)	0.2
Normal 7	0.1	N70 (of BC70)	0.11
Normal 8	0.31	N71 (of BC71)	0.24
Normal 9	0.87	N72 (of BC72)	0.292
Normal 10	1.1	N73 (of BC73)	0.261
Normal 11	0.56	N74 (of BC74)	0.318
Normal 12	0.43	N75 (of BC75)	0.258
Normal 13	0.39	N76 (of BC76)	0.191
Normal 14	0.83		
Normal 15	0.48	geoMean	0.329
Normal 16	0.462	SD	0.3

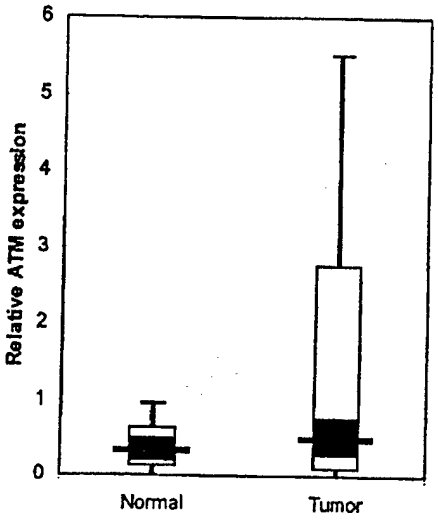
Breast cancer lines	Relative ATM expression	Breast cancer lines	Relative ATM expression
T47D	5.55	MDA468	0.47
MDA435	5.8	MDA361	1.91
MDA231	4.05		
SkBr-3	2.14	geoMean	2.6
MCF7	3.21	SD	1.96

[a]Relative ATM expression in normal breast tissues and breast cancer cell lines.

LOH analysis. The microsatellite markers D11S2179 and NS22 (both intragenic for ATM), D11S1787 (centromeric), D11S1778 (telomeric), D11S1294 (telomeric) and D11S1818 (telomeric) (28,29) were amplified using a standard PCR protocol and ^{32}P-labeled primers. Amplicons were analyzed on 6% acrylamide gels followed by PhosphoImage analysis.

Partial mutational analysis. For mutational analysis, 2 regions of the ATM gene were amplified from the RT-PCR reactions of 8 tumors with the highest ATM expression (BC30, BC66, BC77, BC79, BC80, BC82, BC83, BC84). The first region was identical to the one used for competitive RT-PCR (nucleotide positions 4646-4905 of GenBank #U33841), while the second was in the PI3 kinase domain (nucleotide positions 7980-8310). Products were purified, sequenced in both directions with the

A

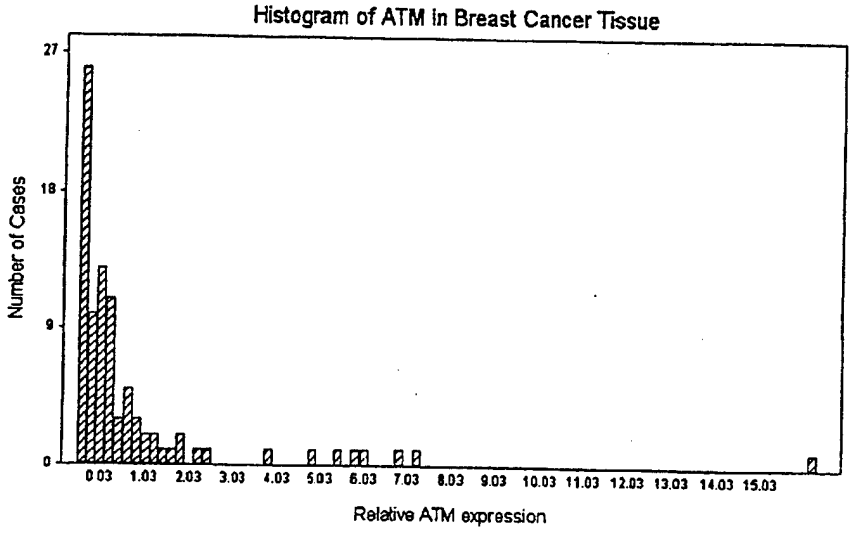


ABI Dye terminator cycle sequencing kit (Perkin Elmer) and analyzed on the ABI sequencer model 377.

Results

ATM expression in breast cancer and normal breast. Using a competitive semiquantitative RT-PCR approach, we determined relative ATM expression levels on 89 breast cancers and compared them to 29 normal breast samples (Table I). Eleven of these constituted matched tumor/cancer pairs. ATM and ß2M transcripts were detectable in all breast tissues and the 7 breast cancer cell lines analyzed. While the expression of ß2M was similar in all samples, ATM expression levels varied widely. Moreover, breast cancer tissues did not show a

B



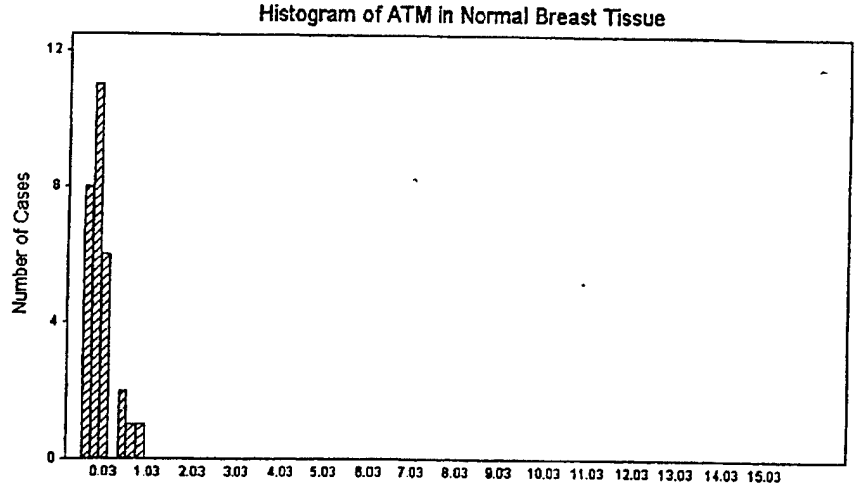


Figure. 1. A, Box plot of relative ATM expression levels in 89 breast carcinomas and 29 normal breast tissues as detected by RT-PCR. ATM expression varies widely in malignant tissue and is 1.5-fold higher on average than in normal tissue. The geometric mean of breast cancer is 0.484±2.5 SD compared to 0.329±0.30 SD in normal breast tissue (p=0.0005). Relative ATM expression in breast cancer range from 0.03 to 16.8 and in normal breast from 0.093 to 1.31. The geometric mean (black bar) with 95% confidence limits (line) and 75% of values (white box) are indicated for both groups. The shaded area represents the 95% confidence interval for the true mean. Values are normalized using the algorithm $ATM_{patient}/ATM_{competitor}/\beta2M_{patient}/\beta2M_{competitor}$. Breast cancer cell lines have the highest ATM levels with a geometric mean of 2.60±1.96 SD and a range from 0.47 to 5.55. B, The same data plotted as a histogram. Step size for the x-axis is 0.2.

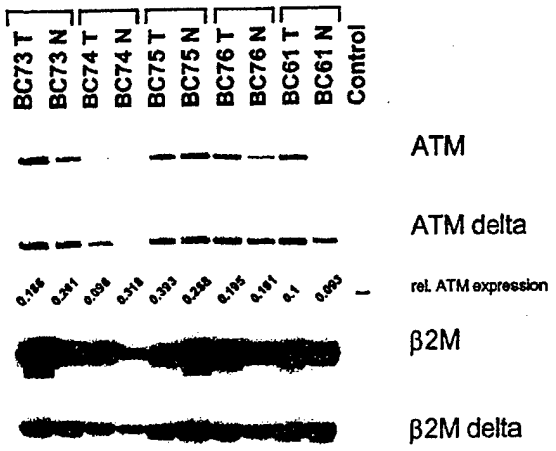


Figure 2. Some examples of raw RT-PCR data for ATM and ß2M mRNA expression. Matched tumor/normal pairs are shown. Numerical values are the relative ATM expression levels after normalization by competitive algorithm. BC74N shows a faint band on the original gel.

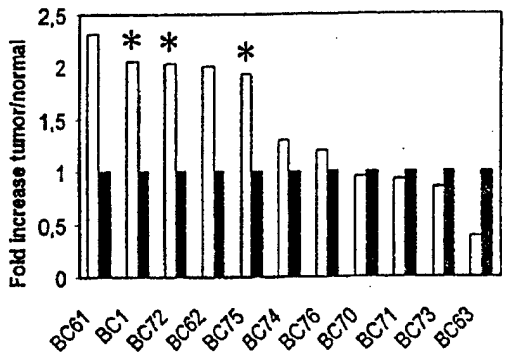


Figure 3. ATM expression levels in 11 matched tumor/normal tissue pairs. Values are normalized using the simple algorithm. White bars tumor, black bars normal. Stars indicate tumors with LOH of the ATM gene. Raw data of some cases are shown in Fig. 2. The overall result was the same when the group was normalized using the competitive algorithm.

Table II. Reproducibility of ATM expression measurements.

Breast cancer case #	Measurement #1	Measurement #2	Average relative ATM expression
BC1	0.15	0.18	0.165
BC12	2.07	2.15	2.11
BC16	0.7	0.81	0.8
BC30	6.25	6.48	6.37
BC33	0.465	0.49	0.478
BC38	0.048	0.056	0.052
BC45	0.05	0.09	0.07
BC49	0.23	0.26	0.25
BC55	0.05	0.05	0.05
BC66	4.3	4.4	4.35
Normal 17	0.14	0.18	0.16
Normal 1	0.62	0.636	0.628

deficiency in ATM expression. Table I contains the complete set of normalized expression data. Fig. 1A shows it graphed as a box plot and Fig. 1B shows the same data plotted as a histogram. In fact, cancers expressed mildly higher (1.5-fold) levels of ATM transcripts than normal breast tissues. However, due to the large variance in breast cancers and the relatively small difference between the geometric means of cancer versus normal tissue, the power to detect significant differences between the two groups was very low. The geometric mean of breast cancer was 0.484±2.5 standard deviation (SD) compared to 0.329±0.30 SD in normal breast tissue (Fig. 1A). In breast cancer, relative ATM expression ranged from 0.03 to 16.8 with a median of 0.57, and in normal breast it ranged from 0.093 to 1.31 with a median of 0.318. Examples of individual raw data are shown in Fig. 2. Repeat determinations from individual patients yielded reproducible results. Table II shows a subset of breast cancers and normal controls with their relative ATM expression levels, averaged from 2 independent measurements of the same sample. A mild tumor-associated increase in relative ATM transcript levels was also seen when the subgroup of matched pairs was analyzed separately. Seven of the 11 normal/cancer pairs showed a 1.2 to 2.3-fold increase in cancers compared to their adjacent normal tissue match, 3 cases were equal and only one case showed decreased (<50%) ATM expression in the tumor (Fig. 3). In line with the findings in primary cancers, breast cancer cell lines had even higher ATM expression with a geometric mean of 2.6±1.96 SD and a range from 0.47 to 5.55.

LOH analysis of 11q22-23. Sixteen matched pairs were analyzed for 6 markers at and around the ATM locus at chromosome 11q22-23. Of these, 2 were intragenic (D11S2179 and NS22), 1 was centromeric (D11S1787) and 3 were telomeric (D11S1778, D11S1294 and D11S1818, in increasing distance) to the ATM gene. Loss of heterozygosity for the ATM markers D11S2179 and NS22 was found in 31% and 25% of the informative cases, respectively. LOH for the extragenic markers was 20% for D11S1778, 20% for D11S1294, 20% for D11S1818 and 33% for D11S1787. Examples are shown in Fig. 4. These frequencies are consistent with those reported in the literature (30,31). Of note is that BC1, BC72 and BC75, all cases with tumor-associated LOH of the ATM gene, expressed higher levels of transcripts in the tumors than in their normal tissue matches (see Fig. 3). This indicates that no correlation exists between tumors with LOH for ATM and decreased expression of the gene product. Since breast tumors with LOH for ATM can in fact overexpress the gene, it renders the possibility of haploinsufficiency less likely. One case with a decreased ATM expression in the tumor could not be assessed for ATM LOH as it was non-informative.

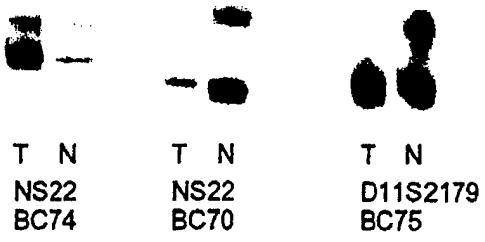


Figure 4. Examples of cases with LOH or retention of heterozygosity within the ATM gene using the indicated markers. N, constitutional DNA; T, tumor DNA.

Partial mutational analysis of the ATM gene. No mutations were found in the 8 tumors with the highest relative ATM expression.

Discussion

In this study, we carried out a comparative analysis of ATM expression in 89 unselected sporadic breast cancers and 29 normal breast tissues. A subgroup of 11 cases comprised matched normal/cancer pairs. The study also included 7 breast cancer cell lines. In contrast to our working hypothesis, we found that cancer tissues did not show a deficiency in ATM expression. In fact, breast cancers expressed 1.5-fold higher levels on average compared to normal breast tissue. Moreover, of the 11 matched cases, 7 cases showed mild tumor-associated increases in ATM expression ranging from 1.2 to 2.3-fold compared to adjacent normal breast tissue. This tumor-associated increase is also reflected in a broad spectrum of breast cancer cell lines (T47D, MDA435, MDA231, MDA468, MDA 361, SkBr-3 and MCF7), which exhibit the highest relative levels of ATM expression compared to normal breast tissue (8-fold). Taken together, our results show that breast cancers and cell lines express somewhat higher ATM levels than normal breast tissues. This effect is independent of previous genotoxic exposure since none of the matched pair patients had received neoadjuvant treatment before surgery. It remains to be shown that the increased ATM levels reflect wild-type transcripts with increased biologic activity. However, this is likely to be the case since our partial mutational analysis was negative and known ATM mutations like those seen in AT patients are mostly truncation and frameshift mutations spread throughout the entire gene. Missense mutations are less common. The amplicon used in this study lies in the middle of the ATM open reading frame (nucleotide position 4646-4905 of 9385 nucleotides). Therefore, it appears unlikely that the majority of our over-expressing samples harbored an unrecognized truncation mutation downstream of the probed region. ATM has been thought of as a constitutive protein and very little is known about the regulation of its expression. ATM protein levels remain unchanged during the cell cycle and after DNA damage by γ-IR (2). However, as seen by the variability and extent of increased expression of some cases in this study, significant regulation seems to occur at the transcriptional level in breast cancer. Interestingly, the proliferative myoepithelial cells in sclerosing adenosis, a benign proliferative disease of breast ducts, exhibit an upregulation of ATM protein, while myoepithelial cells of normal breast have low ATM levels (31). Therefore, a speculative but reasonable interpretation of our results is that the proliferative program of tumor cells elicits a mild upregulation of the ATM gene in breast cancer.

Our ATM measurements for the breast cancer group is in agreement with the ATM levels and variance determined by Waha *et al*, who compared breast carcinomas from 39 patients to normal breast tissues from 4 unmatched control individuals (27). However, the two studies differ in size and values for their control groups (29 controls in this study, of which 11 were matched). Since Waha *et al* found a high ATM expression in their 4 unmatched controls (geometric mean of 5.6), the authors concluded that breast cancers exhibit a reduced ATM level. Therefore, the reason for the different conclusions is unlikely to be technical but could be due to a limited control sample size in the study of Waha *et al*. Of note is that both studies employed the same RT-PCR method including primers and competitors. Taken together, our study does not support the conclusion that decreased ATM expression is specifically associated with neoplastic potential and a reliable marker of breast cancer.

ATM is a member of the phosphatidylinositol-3 kinase family, a conserved family of very large proteins required for a DNA damage-sensitive checkpoint pathway in yeast, *Drosophila* and mammalian cells. Homology is conferred through the carboxy-terminal phosphatidylinositol 3-kinase (PI3-K) domain with 60% homology among family members. A second, cysteine-rich region of lesser homology (50% among family members) is present immediately upstream. ATM is a so-called 'stress kinase' and a DNA strand-break sensor that is involved in the γ-IR induced DNA damage response (33,34), meiotic recombination (35,36) and telomere length monitoring (37). P53 and the non-receptor tyrosine kinase c-abl are important downstream signaling targets for ATM (33,34,38). Moreover, ATM plays a role in p53-independent S and G2/M checkpoints. While ATM exhibits a suppressor role with loss of function mutations in specific sporadic and familial lymphoid malignancies (T-PLL and B-CLL), no convincing evidence for such a role could be shown in sporadic breast cancer despite considerable effort. Consistent with this data, our study shows a lack of defective ATM expression in this disease and therefore does not support a role for ATM in sporadic breast cancer risk or development.

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